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A New Technique For the Histochemical Demonstration of Phosphatase in Hard Tissue

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Introduction

Since the publication of the histochemical demonstration of alkaline phosphatase by Takamatsu (1938) and Gomori (1939), numerous histochemical investigations of phosphatase activities in animal and also human tissues have been reported.

This enzyme will presumably play some important roles on metabolism of tissues. Although many investigations elucidated the biological significances of this enzyme in the metabolism of carbohydrates, lipids, nucleic acids, proteins and minerals, they still seemed to be uncertain. The histochemical works added some new problems to this field. Phosphatase is a common name and probably consists of something like enzymes. The specificity of these enzymes is believed to be the most essential problem of this field.

In regard to ossification, it seems that the old Robison's theory has made an indelible impression on many investigators and some misunderstandings about this enzyme have been current. Takamatsu, one of the present authors, has denied a direct correlation of this enzyme to calcification process of bone formation. In the first study, Takamatsu (1938, 39) has histochemically investigated this enzyme of the bone with undecalcified celloidin sections for the purpose of avoiding the destroying effect of the decalcifying solutions. Kabat and Furth (1941) attempted the decalcification of hard tissues for the histochemical use of this enzyme with a weak acid solution in their first report. Thereafter, decalcifying techniques for the histochemical demonstration of this enzyme in hard tissues with acid solution were developed by Gomori (1943), Bourne (1943), Moag (1946), Lorch (1946), Greep, Fisher and Morse (1947), Lillie (1951) and many other investigators. Although the decalcifying technique with acid solution and reactivation in some alkaline solutions may demonstrate alkaline phosphatase in hard tissues, it doesn't avoid

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the destruction of the enzymatic activities to some extent. Recently some methods for the decalcification with chelating agents in neutral solution were devised by several authors (Dotti et al, 1951; Hahn and Reygadas, 1951; Screebney and Nikifornk, 1951; Birge and Imhoff, 1952; Hilleman and Lee, 1953). In the study of bone tuberculosis, we also devised independently the method with chelating agents as decalcifiers. The abstracts of these studies were already printed in other papers (1954, 1955). Our methods somewhat differ from other author's and moreover, as a result of following Takamatsu-Nishi's new histochemical method for alkaline phosphatase, a clearer pattern will be obtained. Many requests for explanation about our method have arrived. This paper will provide this information. For the experiment of acid phosphatase, the same manner of decalcification may be applicable. The method for acid phosphatase will be described in another paper by the same authors.

Materials and Fixations

The tuberculosis of bones and joints, hitherto, knee-joint, costochondrial junctions of guinea pigs, rats and mice of various ages and also resection specimens from human cases were tested for this experiment. H. Matsuzaki had a test for the tooth of rabbits and other animals and other calcified tissues. All of the hard tissues, also, seemed to be decalcified by the same method.

As the fixatives, 80 per cent alcohol solution, absolute alcohol, pure acetone and a mixture of alcohol and acetone of equal parts were tested. The mixture solution of alcohol and acetone resulted in the best figures.

Decalcification and Reactivation

After the fixation of the hard tissues in a cold mixture of alcohol and acetone about 24 hours, the tissues were split in about 3-5 mm thickness in the sagittal plane and fixed again in the fresh solution of the same mixture for several days. As a control, a kidney slice was also fixed in the same solution and the same procedure was followed. After this fixation, the tissues were washed in water for short time, and then, immersed in a following decalcifying solution at ordinary room temperature.

Decalcifying solution: 5 per cent solution of tetrasodium salt of ethylenediamine tetraacetic acid (EDTA) and 5 per cent solution of disodium salt of EDTA were prepared. These two solutions were mixed in equal parts before use. This mixture indicates neutral nature (about pH 6.8-7.2). The completion of demineralization may be recognized by a daily stab test with a needle,

The demineralization by these chelating agents causes the disappearance of calcium and magnesium ions in the tissue slices. As the magnesium ion is essential for the alkaline phosphatase activities, the loss of the magnesium ion is accompanied with the loss of the enzymatic activity. Accordingly a reactivation treatment by addition of magnesium ion is required. In this revised method, the reactivation took place after sectioning of tissue slices.

After demineralization, the slices were washed in tap water about 30-60 minutes and dehydrated with alcohol, and then, embedded in celloidin as usual. The embedded slices were then sectioned in about 15 micron thicknesses. The celloidin tissue sections can be stored without marked loss of the activity of enzymes in a cooled 90 per cent alcohol for a few weeks. Before histochemical use, these sections were reactivated in a 1 per cent solution of magnesium chloride for several hours.

Histochemical Procedure for Alkaline Phosphatase.

Of late an excellent method for the histochemical demonstration of alkaline phosphatase was devised by Takamatsu and Nishi. This method has also proved its excellence in the experiment of decalcified hard tissues.

The reactivated celloidin sections were tested for the demonstration of alkaline phosphatase as shown in the following.

1) Place in the following mixture of substrates at 37°C for 8-15 hours.

Composition of the mixture :

1 % sodium β -glycerophosphate solution	20 cc
1 % calcium chloride solution	20 cc
Palitzsch's buffer solution	10 cc

This mixture was adjusted to a final pH 9.2 by addition of alkali.

- 2) Wash with distilled water.
- 3) Place in 5 % silver nitrate solution about 5 to 10 minutes.
- 4) Wash with distilled water and place in neutral formol solution about 5 to 10 minutes; in this process, metallic silver will appear by the reduction of silver phosphate.
- 5) Wash with distilled water twice about 20 to 30 minutes and then immerse in 0.1 % gold chloride solution about 1-2 hours; in this procedure, metallic silver will be replaced by gold.
- 6) Wash with distilled water and place in 5 % sodium thiosulfate solution.
- 7) Rewashing under tap water, and counter-stain may be followed by nuclear fast red solution or other suitable dyes.
- 8) Dehydrate through alcohol and mount in balsam as usual.

As a control, some sections from each case were similarly treated except that they were incubated in the mixture without substrate.

Results

The location of alkaline phosphatase activities were shown by deep blue or violet in various grades according to the activities, indicating a formation of metallic gold. At the higher magnifications, the sites were observed as fine granular precipitates in cytoplasm. If the enzymatic activity was very strong, the nuclei were also stained besides cytoplasm. This phenomenon is assumed to be so-called diffusion. Some unsuitable treatments such as an excess of incubation, reactivation and immersion in the silver nitrate solution or in the gold chloride solution may cause nonspecific staining of fibrils or nuclei. The histochemical distribution of the alkaline phosphatase by this method was identical with that of the frozen sections or of the celloidin sections of uncalcified slices which have already been described by Takamatsu et al. For example, the epiphyseal growing zone of normal guinea pig was separated into several regions by the enzymatic activities; the resting cartilage showed activity in neither cells nor matrix, and the outer most proliferating cartilage has slight activity in the oval columnar cells, showing their increasing intensity toward the hypertrophic or maturing cartilage. The calcified cartilage zone was almost free from the enzyme with the exception that a few remaining living cells in it revealed some activity. The osteoblasts among the trabecles of the plate, metaphysis and diaphysis, showed distinct enzymatic activities and the younger osteocytes showed slight activity. The bone matrix was completely negative.

The articular cartilage showed almost no reaction in the outermost cell layer and the interstitial substance, although faint activity was observed in a few oval cells, increasing their intensity toward the inner layer as in the cartilage of epiphyseal plate. The ground substance of compact diaphyseal bone was free from the activity. Osteoblasts and vascular cells at Haversian canals and Volkmann's canals were strikingly positive and the younger osteocytes showed a moderate reaction, while the older and more centrally located osteocytes were negative. The endosteal cells lying close to the compact bone of diaphysis showed marked activity, while the periosteum surrounding the compact bone substance as in the perichondrium showed moderate reaction only in the inner layer and no reaction in the outer layer. The bone marrow cells showed enzymatic activity in various grades of intensity.

In this paper, some results of the staining technique in normal hard tissues only are reported. The findings in various pathological lesions such as experimental bone-joint tuberculosis in the guinea pig and fracture of metatarsus of the rabbit will be described in other reports,

Comments

The revised method for the histochemical detection of alkaline phosphatase in hard tissues consists of three characteristic principles; the first point is the decalcification technique by neutral EDTA solution, the second point is the reduction of silver phosphate, which is formed by the enzymatic reaction, by neutral formol solution and the third point is the substitution of metallic silver for gold in a diluted gold chloride solution. The DETA solution, chelating with metallic ions of two valencies such as calcium and magnesium ion, has the remarkable characteristic of forming soluble complex salts. The neutral solution is available for the decalcification of mineralized tissues without irretrievable loss of enzymatic activities. Various decalcifying solutions such as Lorch's fluid, Greep's fluid, 5 % EDTA (tetrasodium salt) solution and HCl, 5 % EDTA (disodium salt) solution and NaOH, and other mixtures of equal parts of 3 %, 5 % and 10 % tetrasodium salt and disodium salt, were examined as a pretreatment of the detection of alkaline phosphatase.

Consequently the mixture of equal parts of 5 % tetrasodium salt and 5 % disodium salt revealed the most favorable effect (this mixture was adjusted to a pH 6.8-7.2). The decalcifying rate of 5 % neutral EDTA solution was more effective than that of weak acid solutions; Lorch's fluid required four or five times as long a period of decalcification with 5 % neutral EDTA solution at room temperature. In regard to the temperature of the solution, the enzymatic activities were not destroyed at 10-20°C but decreased at over 30°C during the decalcifying procedure.

The decalcified tissues were embedded in celloidin as usual. The final microscopic findings of celloidin sections were clearer and more distinct than that of paraffin sections.

Since the decalcifying procedure brings the retrievable loss of the enzymatic activities, a reactivation treatment must be attended. Excessive reactivation, however, occasionally causes a diffusion phenomenon and an irregular staining in tissue slices. This treatment must be performed adequately but as short a time as possible. For this purpose, 1 per cent magnesium chloride solution was used only in tissue slices in this revised method.

In the staining procedure, the precipitates of tricalcium phosphate which had been produced by the enzymatic reaction were converted into silver phosphate in a silver nitrate solution and reduced to metallic silver using a neutral formol solution instead of sunlight. Then, the metallic silver was substituted for metallic gold by immersing in a diluted gold chloride solution. This metallic gold reveals the distinct blue violet colour and is easily differentiated from various pigments such as lipochrome, hemosiderin

and bile pigment, and this precipitates is stable among many other organic reagents.

Summary

A new histochemical technique for the demonstration of alkaline phosphatase in hard tissues was described. This method is characterized by the following three points. First, a neutral mixture solution of tetra- and disodium salt of ethylen diamine tetraacetic acid is adopted as a suitable decalcifying solution for histochemical use. Secondly, neutral formol solution is applied as a reducing reagent for silver phosphate which is produced by histochemical procedure instead of other Kossa's method. Last of all metallic silver which yielded a reaction product is replaced in metallic gold by immersing in a gold chloride solution.

By this method, the procedure became easier and excellent figures were obtainable.

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(All plates show the distribution of alkaline phosphatase activities)

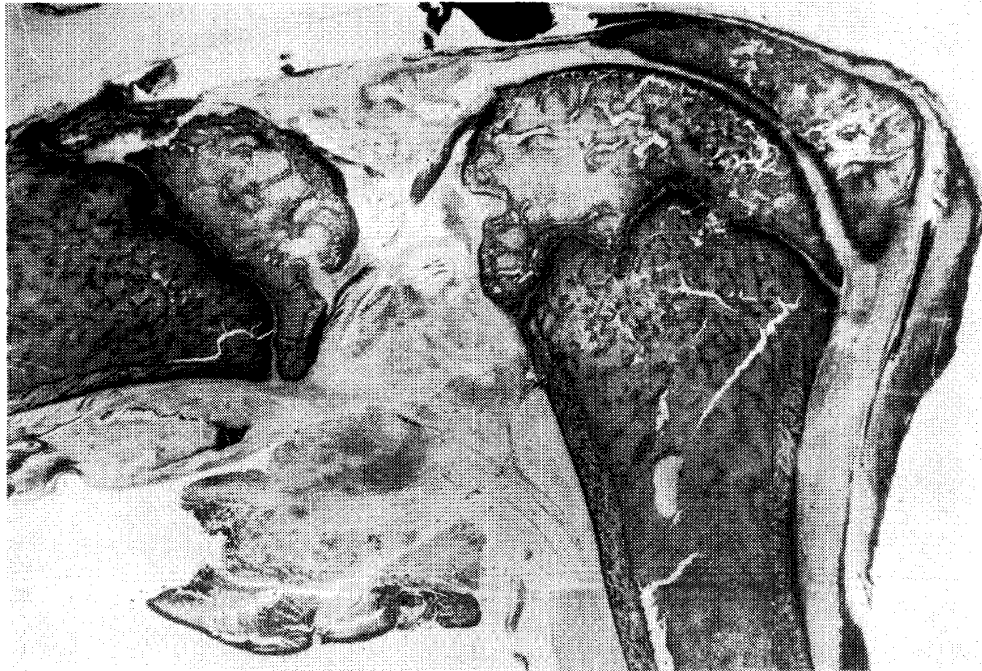


Fig. 1. Longitudinal section of the experimental knee joint tuberculosis of a guinea pig. Two weeks after the inoculation of tuberculous bacilli. Decalcification was completed by immersing in 5 % neutral EDTA solution for 7 days. (×5)

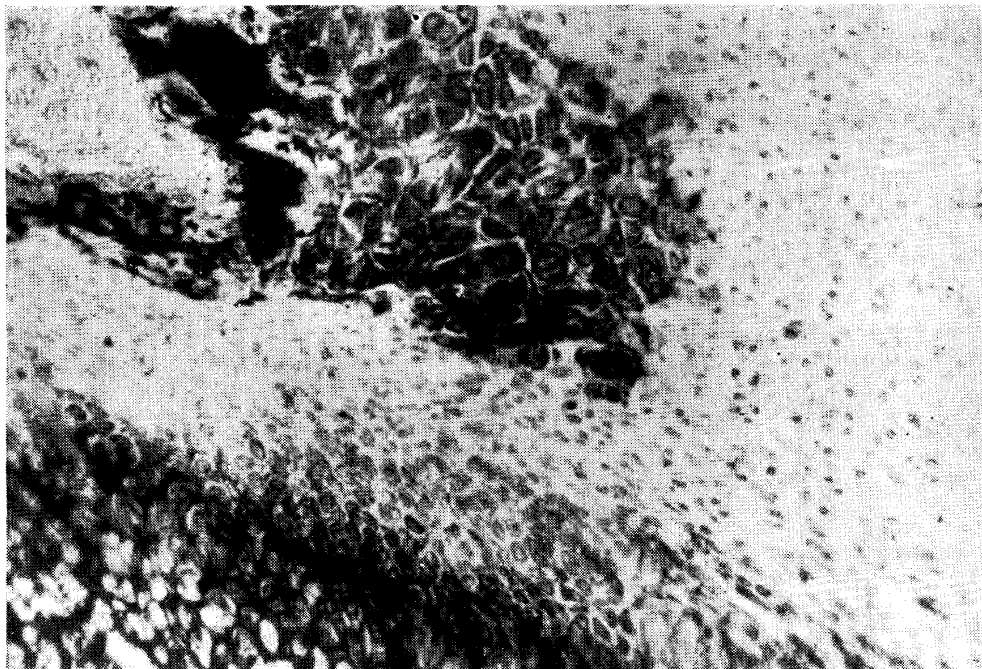


Fig. 2 ; Epiphyseal growing zone and articular cartilage of the distal end of the femur of a normal guinea pig.

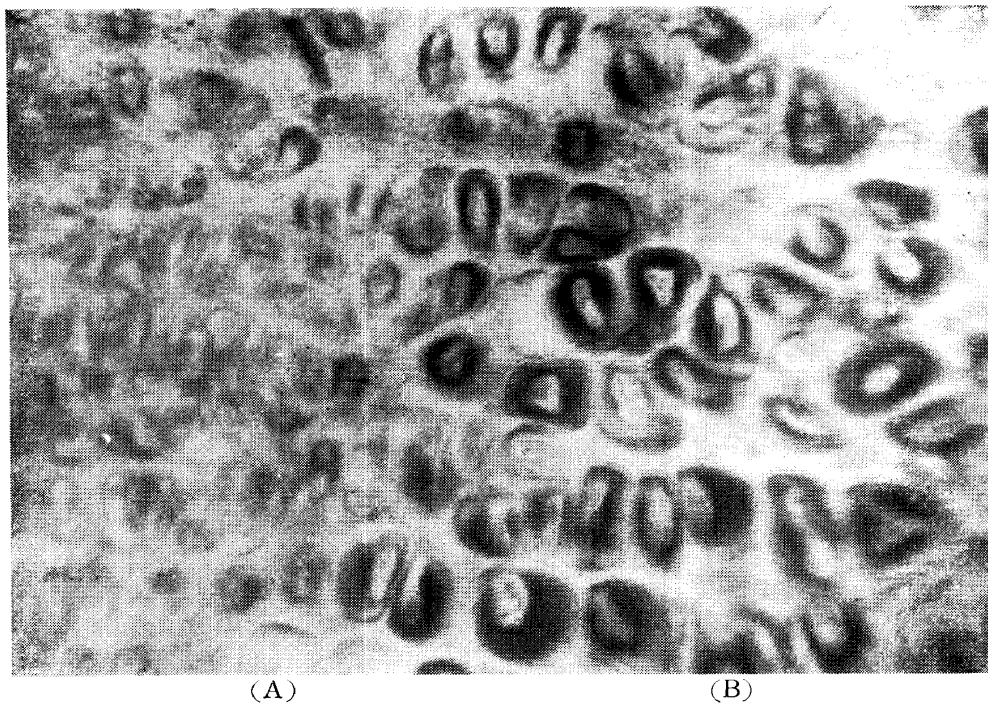


Fig. 3 A high magnification of the epiphyseal cartilage of a young rabbit. In the resting cartilage (A), no or a faint enzymatic reaction is shown. In the proliferating cartilage (B), there is a marked reaction in the cytoplasm. ($\times 500$)



Fig. 4 The kidney of a normal rabbit which was immersed in the decalcifying solution of 5 % neutral EDTA for 5 days. The nuclei were counterstained with nuclear fast red. ($\times 200$)